CARDIOREGULATION IN LIMULUS. I. PHYSIOLOGY OF INHIBITOR NERVES[1]

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Heart muscle contraction in the animal kingdom is initiated by two essentially different mechanisms (Prosser, 1961). In the first type, represented by vertebrate and molluscan hearts, muscle cells initiate the rhythmic contractions (myogenic hearts). In the second type, represented by most adult arthropod hearts, nerve cells initiate the rhythmic contractions, while the muscle cells possess no inherent rhythmicity (neurogenic hearts). Both neurogenic hearts and myogenic hearts are regulated by nervous connections with the central nervous system and, although the basis of the initiation of the contractions is different, a parallelism between the regulatory nerves in neurogenic hearts and those of myogenic hearts can be demonstrated. In both cases an inhibitory set of nerves which decreases and an excitatory set which increases the heart rate are present. Vertebrate (Mitchell, 1956) and molluscan (Welsh, 1953, 1957) cardioregulatory nerves have been extensively investigated, but less information is available on cardioregulatory parameters of arthropods. The most extensive studies have been performed on the hearts of decapod Crustacea (reviewed by Maynard, 1961; and by Florey, 1960).

In a brilliant series of experiments, A. J. Carlson (1905, 1909) characterized the heart of *Limulus polyphemus* as neurogenic. At the same time he demonstrated the existence of both excitatory and inhibitory cardioregulatory nerves. The excitatory nerves have their source in the abdominal ganglia and enter the cardiac ganglion at several points. The inhibitory nerves originate in the last two pairs of nerves leaving the hind-brain and travel in the seventh and eighth pairs of dorsal nerves until they reach the heart at about the fifth or sixth heart segments (Patten and Redenbaugh, 1900; Lochhead, 1950). The original studies of Carlson have not been followed by further detailed studies of the function of the regulatory nerves in *Limulus*. We report here some of the parameters of function of the inhibitory nerves of *Limulus*.

Materials and Methods

Source and maintenance of animals

Animals were obtained from the Supply Department, Marine Biological Laboratory, Woods Hole, Massachusetts. Some experiments were performed at the Marine Biological Laboratory, the majority were carried out at Purdue University. Animals remained healthy for more than six weeks in moist excelsior at ambient temperatures below 21° C.

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Recording

Recordings were made from "excised" preparations by removing the prosomal exoskeleton lateral to the heart on one side and dissecting the seventh and eighth cardiac nerves free of surrounding tissue (see Patten and Redenbaugh, 1900, and Lochhead, 1950, for anatomy and terminology). The anterior two or three segments of the heart were thus visualized. Electrical activity was recorded by means of platinum wire electrodes placed on the cardiac ganglion. Sea water or the saline solution of Chao (1933) (0.44 *M* NaCl, 0.009 *M* KCl, 0.037 *M* $CaCl_2$) were used to supplement the blood.

The method described above has drawbacks. (1) The stretch normally applied to the heart by the supporting ligaments is destroyed. (2) The normal flow of blood into the heart during diastole is destroyed. Both of these factors are known to influence the heart inotropically and chronotropically (Carlson, 1907). For these reasons a method of recording heart rate from the intact animals has been developed:

A 1 × 1 cm. piece of dorsal exoskeleton, directly over the heart in the region of the sixth heart segment, was removed. With care, removal without damage to the underlying hypodermis and without loss of blood is possible. Small-gauge insulated platinum electrodes were then inserted through the epidermal tissue to the cardiac muscle or ganglion. Placement of the electrodes was checked by the correlation between the electrical activity and the movement of the heart, as indicated by the simultaneous movement of the overlying hypodermal tissue. If visual monitoring was not needed, electrodes were simply passed through small holes drilled into the carapace overlying the heart.

Electrical activity was amplified with an A.C. preamplifier, displayed on an oscilloscope, and recorded simultaneously with a magnetic tape recorder.

Mechanical recording

In addition to measurement of the electrical activity, records of the mechanical movement of the heart are desirable. This is especially true when electrical stimulation is used since it causes interference with the electrical record. For such recordings a 3-0 stainless steel insect pin was bent into a hook, a small hole drilled in the same region used for the electrical recordings, and the pin inserted through the epidermal tissue and hooked into the dorso-lateral heart muscle. The pin was connected to a Statham force transducer. The small opening in the epidermal tissue, the low blood pressure of the animal and the quick clotting time of the blood prevent any great loss of blood while performing these operations.

Stimulation of inhibitory nerves.

Inhibitory nerves were stimulated with a square-wave stimulator *via* stainless steel insect pins or platinum wires. Such stimulation is complicated by the fact that it has been impossible completely to isolate the heart from the animal without disrupting connections of the inhibitory fibers to the cardiac ganglion. Previous workers have compromised and dissected the heart as free as possible from the animal, but we feel that it is desirable to record heart contractions in an animal

which has been manipulated as little as possible. We, therefore, place most confidence in the studies employing "intact" preparations. For these, stimulation was accomplished with electrodes penetrating the ventral integument medial to the first branchiothoracic muscle and posterior to the last pair of walking legs. In this region the inhibitory fibers anastomose and pass dorsally toward the heart.

The stimulus was normally given for a period of 30 seconds, followed by a 100-second rest interval. The sequence in which the various frequencies were presented was randomized to eliminate cumulative effects.

Results

Normal heart rates

To establish a control value for the rate of beat, we have placed hearts excised from *Limulus* in sea water or in saline solution for five hours or more and have monitored their rates of beats continuously during this period. From the direct

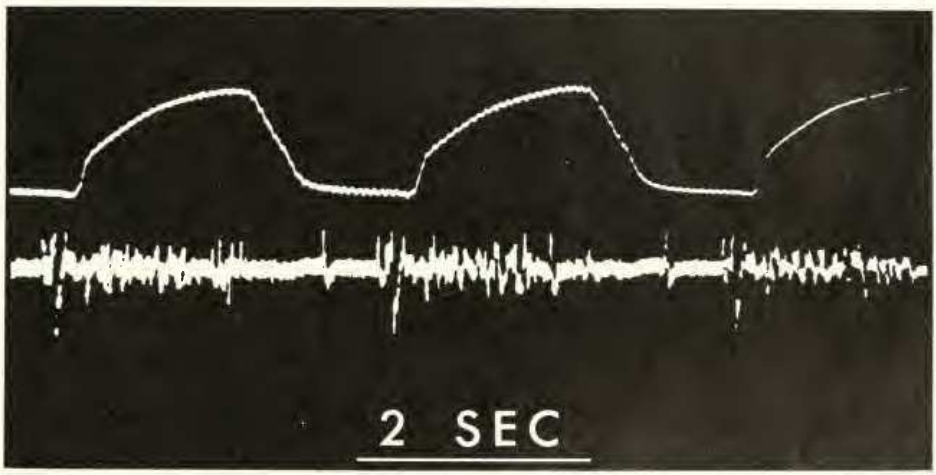

Figure 1. Normal electrical activity (lower record) and muscular contractions in an isolated heart (upper record). Upward deflection indicates contraction.

recordings so obtained, cursory monitoring disclosed no marked changes in rate over this period. A section of such a record is shown as Figure 1.

Sample intervals between ten contractions, chosen by use of a table of random numbers, from recordings of five animals over long periods were chosen, the instantaneous rates calculated and the mean frequency of beat and standard deviation were obtained by standard statistical techniques. The values so obtained were compared by a brief analysis of variance to determine the confidence which we could place in our further measurements.

Data for five hearts summarized in Table I show that while the mean rates so calculated range between 17.26 and 27.61 beats per minute, the variation in the rate for any one individual is small, as may be judged by the low standard deviation (1.89 compared with a mean value of 21.58 for all animals) of the rates.

The same analysis indicates that the variability between animals is so large ($s = 12.28$) that large numbers of animals would be required to make valid quantitative comparisons between animals. There is no correlation between heart rate and size, sex or season.

The heart beat of intact preparations can be monitored for several days. Direct observation for up to 24 hours showed no marked changes in rate of beat. Measurements similar to those carried out on the excised hearts disclosed mean rates ranging from 14.90 to 23.25 beats per minute. Once more, a comparison of the standard deviations shows that there is little point in comparing rates among individuals, but that the low standard deviation ($s = 0.364$) of the variability in rate of any one individual permits us to attach considerable significance to changes in rate greater than about one beat per minute.

Effect of inhibitory nerve activity

Stimulation of the inhibitory fibers of excised preparations resulted in a slowing of the rate. The magnitude of this slowing was dependent on stimulus strength

TABLE 1

Observations on 5 intact hearts and 5 excised hearts. Values are in beats per minute

	Intact	Excised
	17.17	22.18
	14.90	17.26
	23.25	21.49
	17.29	27.61
	20.90	19.35
$\bar{X}$	18.70	21.58
Standard Deviation (Intra-animal)	0.364	1.89
Standard Deviation (Inter-animal)	10.52	12.28

and frequency. In a typical preparation, stimulus strengths of four volts or less (20 cycles/sec., 1-msec. duration) were ineffective while six volts or more caused a significant decrease in rate. In four other animals the minimum effective stimulation was between three and seven volts. Raising the stimulus strength above this threshold value resulted in no further increase in the effectiveness of stimulation. It therefore appears that all the inhibitory fibers are brought into action within a narrow range of stimulus strengths. Either there are comparatively few inhibitory fibers or they all have very nearly the same electrical characteristics.

In the intact animal the relationship of change in heart rate to stimulus strength is essentially similar to that found in the excised preparations. Figure 2 is a typical record. In a typical case, a stimulus strength above 40 volts (20 cycles/sec., 1 msec.) was required before any significant slowing of the heart rate was noted. In five intact preparations, the threshold ranged from a low of about 20 volts to a maximum of slightly over 100 volts. The higher threshold in intact preparations is undoubtedly due to shunting of the stimulating current.

In a series of four intact preparations a study of the relationship between stimulus frequency and decrease in heart rate was made. Because of the variability

among animals, discussed above, we have calculated the relative rate of contraction for each animal and compared these changes. We define the relative rate as the minimum rate of beat during stimulation, divided by the pre-stimulation rate for the same animal. Figure 3 shows the results of these experiments. The relationship between stimulus frequency and relative slowing of the heart is not linear. Maximum slowing of the heart is achieved by stimulus frequencies between 10 and 80 cycles/sec., although stimulation at frequencies as low as 2.5 cycles/sec. caused some slowing. At a frequency of stimulation of 200 cycles/sec. there was an increase in heart rate.

The response of the heart is not tightly coupled with the onset of stimulation of the inhibitory fibers. At the beginning of a period of stimulation the heart rate sometimes becomes gradually slower over a period of 10 to 15 seconds, *i.e.*, the

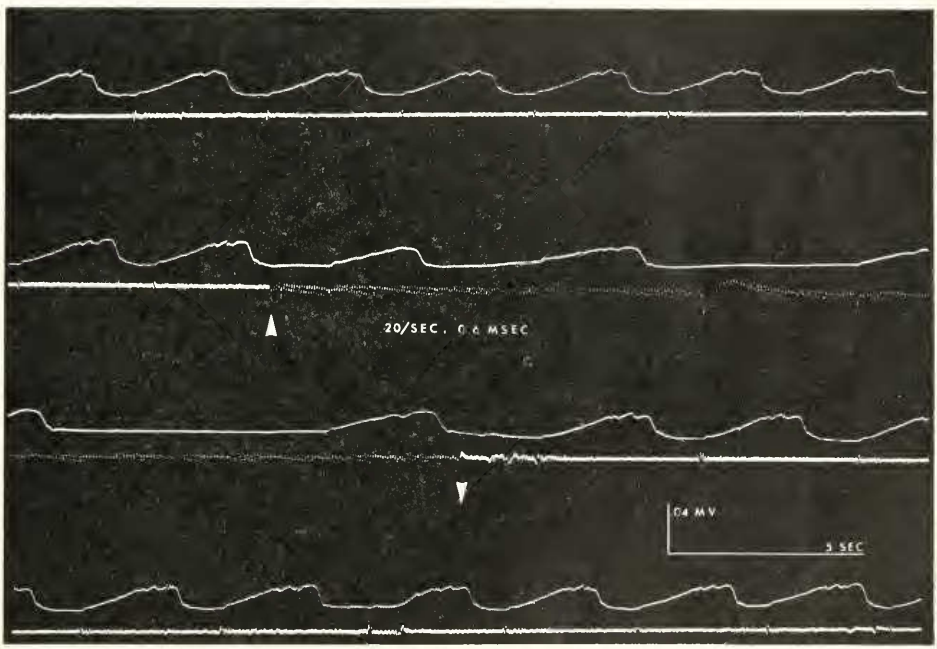

FIGURE 2. Changes in heart rate in an intact animal when inhibitory nerves are stimulated. The record is continuous from top to bottom. The upper record in each case is muscular contraction with increasing tension an upward deflection. The stimulus begins at the upward arrow and stops at the downward arrow.

time interval between beats becomes progressively greater for two or three beats before becoming stable at some new level. In a similar manner the end of stimulation and return of the rate to the normal level are not simultaneous. Half a minute is sometimes required before the rate has again returned to the prestimulation rate. This represents 5 or 6 beats in which the time interval between beats is becoming progressively less.

If the inhibitory nerves are stimulated at some constant frequency for a period of 30 seconds, with intervening rest periods of 100 seconds, the maximum response

is not constant from one stimulation period to the next. Instead, at times the heart may slow by as much as 60% but at other times slow by less than 1%. Figure 4 shows a typical case. This also indicates that there is no tight coupling between activity in the inhibitory nerves and heart rate. There was no apparent correlation between the relative decrease in rate with stimulation and the number of previous stimulations.

Discussion

These experiments confirm the earlier observations of Carlson (1909) on the cardioinhibitory functions of extrinsic nerves in *Limulus*. They also extend our

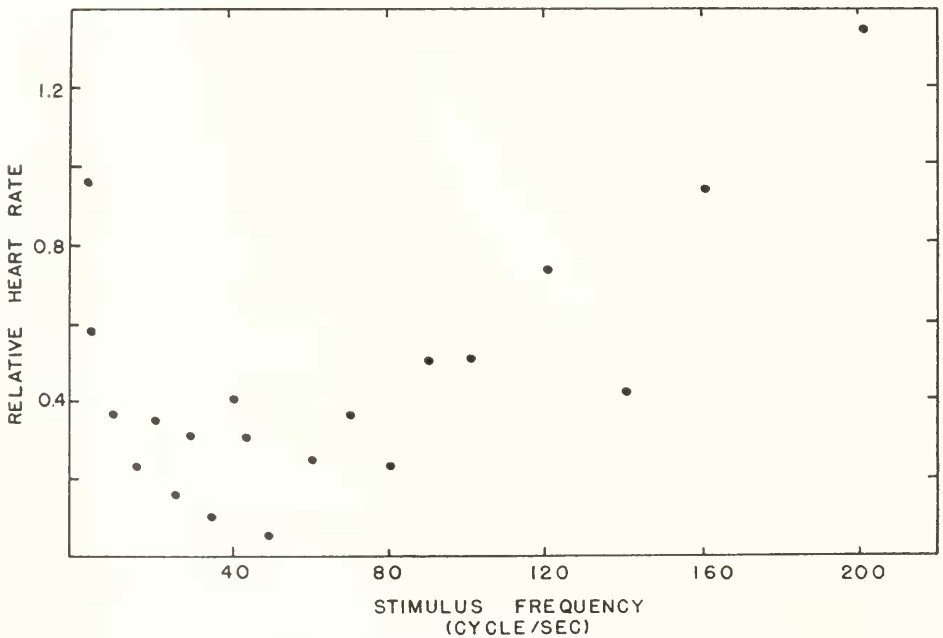

Figure 3. Relationship between heart rate and frequency of stimulation of inhibitory nerves in intact animals. Each point represents the average for four animals. Stimuli were at suprathreshold value and 1-msec. duration for 30 seconds. The average rate was calculated for the 30-second period.

knowledge of some of the parameters of cardioinhibition in *Limulus*, since Carlson was unable to obtain quantitative results with available equipment.

The range of heart rates, from 14.9 to 27.5 per minute, which we observed are within the range of 8 to 28 previously reported for *Limulus* (Spector, 1956). There is little or no difference in the rate of beating of hearts in intact animals and the "excised" preparations. It therefore seems likely that the cardioregulatory nerves are probably not continuously biasing the heart rate but rather that they function only intermittently.

The range of heart rates which occur in *Limulus* makes quantitative comparisons of induced changes in rate between animals difficult. Since the rate for any particular animal varies only within narrow limits it is feasible to obtain quantitative results if we use one animal's heart rate as its own control. For example, in intact animals rate changes as small as one beat per minute can be assigned significance.

The graded responsiveness of the heart rate to stimulations of increasing frequencies is worth comparing with the results of similar experiments performed on the crayfish (Florey, 1960). In that crustacean the change in heart rate was also found to be a function of the frequency of stimulation of the inhibitory fibers. However, the response differed from that found in *Limulus*. In the crayfish, frequencies

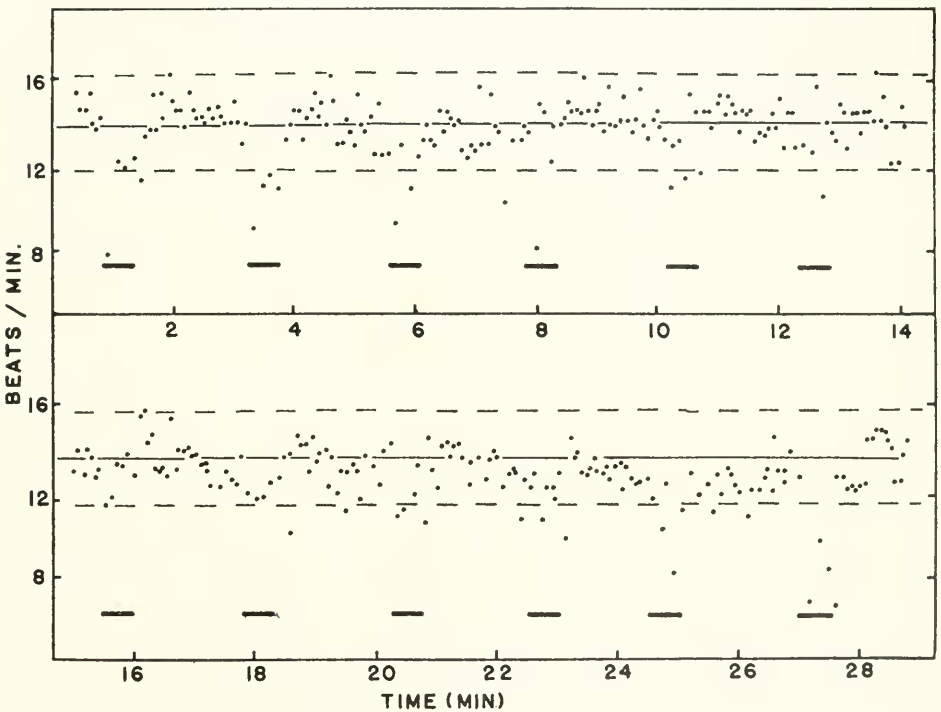

FIGURE 4. Variability in heart rate with constant stimuli. Stimuli were at 20 cycles/sec., 1-msec. duration for 30 seconds. Periods of stimulation are shown by the heavy horizontal lines at the bottom. The frequency of heart beat is in beats/min. Each point represents one beat. The solid horizontal line is the mean unstimulated rate and the dotted lines are three standard deviations on either side of the mean.

of stimulation less than 15 cycles/sec. are ineffective while frequencies greater than 35 cycles/sec. result in complete standstill of the heart. In the range of frequencies of stimulation between 15 and 35 cycles/sec. the decrease in heart rate rises almost exponentially as the frequency of stimulation is increased.

In *Limulus*, frequencies of stimulation of 2.5 cycles/sec. cause some slowing in the heart. As the frequency is increased, the heart rate does not continue to

decrease but rather reaches a low point by 15 cycles/sec. and remains at this level until a frequency of stimulation of almost 90 cycles/sec. is reached. Frequencies above 90 cycles/sec. are less effective in slowing the heart than those below that level. At a frequency of stimulation of 200 cycles/sec. there is an actual increase in the heart rate. Carlson (1905) reported that with proper adjustment of the stimulus frequency and strength he was able to produce complete standstill of the *Limulus* heart. We were not able to duplicate these results.

From the differences in the response of the crayfish heart and the *Limulus* heart, it seems likely that in *Limulus*, but not in the crayfish, some excitatory fibers are contained in the inhibitory nerve. Since stimulation of the seventh and eighth nerves at high frequency causes a cardiac acceleration, the net effect of nerve activity may result from a combination of excitatory and inhibitory activity. At low frequencies of stimulation the inhibitory fibers predominate, but at frequencies above 90 cycles/sec. either the inhibitory fibers become less effective or the excitatory fibers more efficient. At a stimulation frequency of 200 cycles/sec. the excitatory fibers predominate. At the site of stimulation, the inhibitory nerves are mixed, carrying fibers which innervate leg and gill musculature as well as the cardiac ganglion. For this reason we believe that further studies of the effects of variations in the frequency of stimulation require the identification of the nerve in a region where it consists largely or exclusively of cardioregulatory fibers.

Summary

1. A method of monitoring heart function in the intact *Limulus* is described.

2. Isolated hearts had rates ranging from 17 to 28, with a mean of 21.6 beats/min. The rate for any individual heart remained nearly constant ($s = 1.89$) over a period of hours.

3. Heart rate in intact animals ranged from 15 to 23, with a mean of 18.7 beats/min. The rate for any individual animal remained nearly constant ($s = 0.36$) over a period of hours.

4. Electrical stimulation of the last two pairs of nerves leaving the hindbrain causes a slowing in heart rate which is dependent on stimulus strength and frequency. Maximum slowing occurs with stimulation frequencies between 10 and 80 cycles/sec. and a lesser slowing at frequencies outside this range.

5. Changes in heart rate are not tightly coupled to stimulation of the inhibitor nerves. A time lag in response occurs both at the beginning and the end of stimulation periods.

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